PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/04, C12Q 1/68, G01N 33/574 — A1

(11) International Publication Number: **WO 96/38463**

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/US96/08235

(22) International Filing Date: 31 May 1996 (31.05.96)

(30) Priority Data:
08/455,896 31 May 1995 (31.05.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/455,896 (CIP)
Filed on 31 May 1995 (31.05.95)

(71) Applicant *(for all designated States except US)*: WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WATSON, Mark, A. [US/US]; 1461 Meadowside Drive, St. Louis, MO 63146 (US). FLEMING, Timothy, P. [US/US]; 743 Eaglebrooke Drive, St. Louis, MO 63021 (US).

(74) Agents: HOLLAND, Donald, R. et al.; Howell & Haferkamp, L.C., Suite 1400, 7733 Forsyth Boulevard, St. Louis, MO 63105-1817 (US).

(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: DNA SEQUENCE AND ENCODED MAMMARY-SPECIFIC BREAST CANCER PROTEIN

(57) Abstract

A purified and isolated DNA sequence and the encoded mammary-specific protein, mammaglobin, are disclosed. Also disclosed are methods for the detecting breast cancer based upon the overexpression and secretion of mammaglobin by breast cancer cells. The methods detect and/or quantitate the presence of mammaglobin or the mRNA encoding mammaglobin.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

DNA SEQUENCE AND ENCODED MAMMARY-SPECIFIC BREAST CANCER PROTEIN

Background of the Invention

(1) Field of the Invention

This invention relates generally to the field of breast cancer pathogenesis and, more particularly, to a cDNA sequence and encoded mammary-specific protein for use in detecting and treating breast cancer.

(2) Description of the Related Art

Breast cancer is one of the most common and potentially lethal of cancers. Although early diagnosis and treatment can reduce morbidity and mortality related to the disease, the positive predictive value of mammography has been estimated to be only about 25% (Hall et al., *N Engl J Med* 327:319-328, 1992 which is incorporated by reference). It would, therefore, be desirable to have a means for detecting the cancer earlier than the cancer can be detected using mammography and a genetic or biochemical marker might be able to provide such means to complement and increase the predictive value of mammography. (Hayes, *Hematol Oncol*

Clin N Am 8:485, 1994 which is incorporated by reference).

The development of breast cancer is accompanied by a number of genetic changes (For review see Porter-Jordan, *Hematol Oncol Clin N Am* 8:73, 1994 which is incorporated by reference). Such changes include gross chromosomal alterations and loss of genetic markers (Devilee et al, *Biochim Biophys Acta 1198*:113, 1994; Callahan et al, *J Cell Biochem Suppl 17*:167, 1993 which are incorporated by reference). The progression of breast neoplasia has also been shown to result in qualitative and quantitative changes in expression of previously identified genes that encode growth factors and their receptors (Zajchowski et al., *Cancer Res 48*:7041, 1988 which is incorporated by reference), structural proteins (Trask et al., *Proc Natl Acad Sci 87*:2319, 1990 which is incorporated by reference), second messenger proteins (Ohuchi et al., *Cancer Res 26*:2511, 1986 which is incorporated by reference), and transcription factors (Harris, *Adv Cancer Res 59*:69:1992 which is incorporated by reference). These changes in gene expression could potentially form the basis for developing a breast cancer marker, although the precise role of these gene changes in the pathogenesis of breast carcinoma in patient biopsy samples is not well understood.

In addition to providing a genetic or biochemical marker for breast cancer for early detection of the disease, it would also be desirable to have a tumor marker that might provide an estimation of prognosis, a means for selection and evaluation of therapy and a means for the targeting of therapy. Although a number of tissue markers have been identified, none are sufficiently sensitive or tumor specific to be ideally suited for diagnosis or for screening the general population. (*Id.*). Thus, there remains a continuing need

for a breast cancer marker such as a gene along with its expressed protein that can be used to specifically and selectively identify the appearance and pathogenic development of breast cancer in a patient.

Using a modified differential display polymerase chain reaction technique to isolate differentially expressed sequence tags from mammary carcinoma, several sequence fragments were isolated that were uniquely expressed in neoplastic mammary epithelial tissue as compared to normal tissue controls (Watson and Fleming, *Cancer Res* 54:4598-4602, 1994 which is incorporated by reference). The discovery of one of these sequence tags identified as DEST002 has led to the discovery and isolation of the novel full length cDNA and encoded protein now referenced as mammaglobin. The cDNA and protein are both new.

Summary of the Invention

Briefly, therefore, the present invention is directed to the identification of novel genes whose expression is increased in breast cancer and to the isolating of cDNA's from the mRNA's of these genes. Accordingly, applicants have succeeded in discovering a novel cDNA and the encoded mammary-specific secretory protein, mammaglobin. The cDNA is in purified and isolated form and identified as SEQ ID NO:1 and the encoded protein, mammaglobin is in purified and isolated form and identified as SEQ ID NO:2.

Mammaglobin is overexpressed in 27% of stage I primary breast cancer tumors. This suggests that dysregulation of the mammaglobin gene occurs early and frequently in breast cancer. The discovery of mammaglobin and its cDNA, therefore, provide the basis for the development of novel methods and compositions for the d tection and treatment of breast neoplastic disease in humans and other mammals.

Thus, the pr sent invention is also directed to novel methods for detecting the presence of breast neoplasia cells in a sample. In one embodiment cDNA encoding mammaglobin or a derivative of said cDNA is used to detect the presence of mammaglobin mRNA in a sample. The method comprises the steps of: (a) providing a polynucleotide containing a nucleotide sequence having the sequence of SEQ ID NO:1 or a derivative thereof, (b) incubating the nucleotide sequence with the sample under conditions in which the sequence can hybridize with mRNA from breast neoplasia cells, and (c) detecting the existence of a DNA-RNA hybridization complex.

Another aspect the present invention provides for a kit for detecting the presence of breast neoplasia cells in a sample. The kit comprises a polynucleotide containing a nucleotide sequence having the sequence of SEQ ID NO:1 or a derivative thereof packaged in a container.

In another embodiment of the present invention, mammaglobin or a derivative thereof is used to detect the presence of cDNA that is reverse transcribed from mammaglobin mRNA in a sample. The method comprises the steps of: (a) producing a cDNA from mRNA using the reverse transcription method in a sample obtained from a patient, (b) providing two oligomers which are primers for the polymerase chain reaction method and which flank or lie within a cDNA encoding mammaglobin, and (c) amplifying the cDNA encoding mammaglobin by the polymerase chain reaction method. The two oligomers comprise SEQ ID NO:3 and SEQ ID NO:4.

Another embodiment to the present invention provides a kit for detection of the presence of breast neoplasia cells in a sample. The kit comprises two oligomers which are primers for the polymerase chain r action method and which are flank or lie within a cDNA encoding mammaglobin packaged in a container. The two

oligomers comprise SEQ ID NO:3 and SEQ ID NO:4.

In another embodiment of the present invention, the presence of the mammaglobin expressed by a tumor cell is detected in a sample using specific antibodies to the protein, mammaglobin. The specific antibodies can be polyclonal or monoclonal antibodies.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of a nucleotide sequence and encoded amino acid sequence that can serve as markers for breast cancer cells; the provision of methods for early detection of the presence of breast neoplasia cells; the provision of means for detecting breast cancer that can complement mammography and increase the predictive value; and the provision of methods that can provide an estimation of prognosis; and the provision of markers that will allow the targeting of therapy.

Brief Description of the Drawings

Figure 1 illustrates the strategy used to isolate the full length mammaglobin cDNA using the Rapid amplification of cDNA Ends (RACE) Polymerase Chain Reaction (PCR) technique and subsequent subcloning into vectors pGEM7Z and pCEV27.

Figure (2) illustrates the human cDNA sequence of SEQ ID NO:1 (nucleotides numbered above) and the amino acid sequence of the encoded the mammary-specific protein, mammaglobin (SEQ ID NO:2)(amino acids numbered below), the solid bar illustrating the 403 bp fragment (SEQ ID NO:5) isolated by the RACE PCR method and the open bar indicating the 206 bp DEST002 sequence (SEQ ID NO:6);

Figure 3 illustrates the amino acid sequence of the mammary-specific protein, mammaglobin (hMAM), (SEQ ID NO:2) compared to rat prostatic steroid binding protein subunit C3 (rPSC3)(SEQ ID NO:7) and human clara cell 10 kD protein (hCC10)(SEQ ID NO:8) with identities marked by

bold letters and double lines and structurally similar amino acids marked by single lines;

Figure 4 illustrates (A) the Northern blot analysis of hybridization of the human cDNA sequence encoding the mammary-specific protein, mammaglobin (hMAM), to mRNA expressed by tissues from breast neoplasia, normal breast and other adult tissues and (B) the analysis of RT/PCR amplified samples of tissues from breast neoplasia, normal breast and other adult tissues;

Figure 5 illustrates the translation of the mammary-specific cDNA sequence in an *in vitro* rabbit reticulocyte lysate assay system;

Figure 6 illustrates Northern blot hybridization with the cDNA encoding mammaglobin detecting mRNA in tumor 2410, in tumors from three of eight other patients (shown in bold), and to a lesser extent, in normal breast tissue (shown in italics) comparing in two cases, mammaglobin expression in tumor tissue and patient matched normal tissue;

Figure 7 illustrates the Western blot analysis using polyclonal antibody to the 16 C-terminal amino acids (SEQ ID NO:14) from conditioned medium (S) and cell lysate (C) from MDA-MB-415 breast tumor cells in the absence (-) and presence (+) of the peptide used to generate polyclonal antibody;

Figure 8 illustrates the Western blot analysis of cell lysates from human breast tumor cells showing detection of mammaglobin protein using polyclonal antibody to the 16 C-terminal amino acids (SEQ ID NO:14) and goat anti-rabbit antibody visualized by enzyme-linked chemoluminescence;

Figure 9 illustrates in color a paraffin-fixed section of breast cancer cells from a patient specimen immunohistochemically stained using polyclonal antibody to the 16 C-terminal amino acids (SEQ ID NO:14) and goat anti-rabbit antibody tagged with horseradish peroxidase

and DAB as substrate showing a brown staining of cells expressing the mammaglobin protein;

Figure 9A illustrates in black and white a paraffin-fixed section of breast cancer cell from a patient specimen immunohistochemically stained using polyclonal antibody to the 16 C-terminal amino acids (SEQ ID NO:14) and goat anti-rabbit antibody tagged with horseradish peroxidase and DAB as substrate wherein the brown staining of cells expressing the mammaglobin protein is indicated.

Description of the Preferred Embodiments:

One aspect of the present invention is based upon the identification and sequencing of the cDNA identified as SEQ ID NO:1 which encodes a mammary-specific secretory protein, mammaglobin, identified by SEQ ID NO:2 (FIG 2). As described below, the full length mammaglobin cDNA was isolated starting from tumor cell mRNA that was reverse transcribed, amplified using the technique of PCR and subcloned into expression vectors. In addition, the protein, mammaglobin, encoded by the cDNA was identified and characterized.

Using the anonymous sequence tag previously designated DEST002, it was demonstrated that the corresponding gene product, which was up until now unknown but herein identified as mammaglobin, is particularly abundant in the breast cancer tumor cell line MDA-MB-415. To isolate the full length mammaglobin cDNA, the mRNA was reverse transcribed from this cell line and cloned using the RACE PCR technique (Edwards et al. *Nucleic Acids Research 19*:5227-32, 1991 which is incorporated by reference). This technique is based upon the strategy of ligation of single-stranded oligodeoxyribonucleotide to the 3' end of single-stranded cDNA. The method by which the mammaglobin cDNA was isolated is represented schematically in FIG 1. The full

length 503 bp cDNA sequence (SEQ ID NO:1) was deduced from the sequence information obtained from the 403 bp fragment (SEQ ID NO:5) (FIG 2) isolated by this technique along with sequence information previously obtained from the corresponding DEST sequence (DEST002, SEQ ID NO:6) in our earlier study (Watson and Fleming, *supra*) (FIG 2). The full length mammaglobin cDNA and the encoded polypeptide is shown in FIG 2. Within the 503 bp cDNA is a 279 bp open reading frame which encodes a polypeptide of 93 amino acids and predicted molecular mass of 10.5kD (FIG 2). The first 19 residues of this open reading frame also predict a hydrophobic peptide signal sequence. The initial methionine of the open reading frame contains a near-perfect Kozak consensus sequence (Kozak, *Cell* 22:7-8, 1980 which is incorporated by reference). The 60 bp upstream of this sequence contain no other in-frame methionines or translational stops. The 3' untranslated sequence of the cDNA constitutes 163 bp and contains a polyadenylation signal, AATAAA, 12 bp upstream of the priming site of the original DEST002 sequence. These data indicate that the full length mammaglobin cDNA has been isolated.

A search for DNA sequences similar to the mammaglobin cDNA sequence in Genbank using the BLAST algorithm (Benson et al., *Nucl Acid Res21*:2963-2965, 1993; Altschul et al, *J Mol Biol 215*:403-410, 1990 which are incorporated by reference), identified no obvious DNA sequence homologies. Thus, mammaglobin cDNA is believed to be a novel, heretofore unknown DNA sequence.

A search of other polypeptides for sequences related to mammaglobin revealed an amino acid sequence homology between mammaglobin and other polypeptides. Mammaglobin exhibited 42% amino acid identity (58% including conservative substitutions) with rat prostatic steroid binding protein (prostatein) subunit C3 (rPSC3) (FIG 3) (SEQ ID NO:7). Rat prostatic steroid binding

protein is a major secretory protein in the rat ventral prostate consisting of a tetrameric protein composed of two different dimeric subunits; C3/C1 and C3/C2 (Parker et al., *Ann N Y Acad Sci 438*:115-124; Parker et al., *J Steroid Biochem 20*:67-71, 1984 which are incorporated by reference). The C1, C2, and C3 genes all encode approximately 6 kD secretory proteins and are thought to have arisen from gene duplication, but while the C1 and C2 genes show strong homology to each other, they are much less similar to the C3 gene. Correspondingly, mammaglobin shows no sequence homology with the C1 or C2 proteins.

As noted above, prostatic steroid binding protein (prostatein) is the major secretory protein in the rat ventral prostate and its expression is regulated by androgenic steroids (Parker et al, *Ann N Y Acad Sci 438*:115-24, 1984; Parker et al, *J Steroid Biochem 20*:67-71, 1984 which are incorporated by reference). Another protein, human estramustin-binding protein (hEMBP) has been reported to be expressed in human prostate, human breast cancer and human malignant melanoma. (Bjork et al, *Cancer Res 42*:1935-1942, 1982; Bjork et al, *Anticancer Res 11*:1173-82, 1991 which are incorporated by reference). Human estramustin-binding protein is immunochemically similar to rat estramustin-binding protein, which has been postulated to be identical to rat steroid-binding protein, prostatein. As noted above, the amino acid sequence of mammaglobin exhibited 42% amino acid identity and 58% homology including conservative substitutions with the C3 subunit of prostatein. Thus it is possible that mammaglobin could be in some way related to hEMBP. However, while both prostatein and hEMBP are detected in the prostate gland, mammaglobin mRNA is completely absent in this tissue. Hence, mammaglobin is neither th same protein nor a subunit of hEMBP and, furthermore, the sequence of hEMBP has not been

determined so that it is not known whether there is even any similarity of mammaglobin with some fragment or subunit of hEMBP.

Although recent reports have demonstrated the rPSC3 promoter fused to SV40 T antigen produces both prostatic and mammary carcinomas in transgenic mice (Maroulakou et al., *Proc Nat Acad Sci U.S. 91*:11236-11240, 1994; Sandmoller et al, *Oncogene* 9:2805-2815, 1994 which are incorporated by reference), the true biological function of this protein is unknown. Furthermore, notwithstanding the hypothesized relationship of rat prostatic steroid binding protein to human EMBP, no human polypeptide or human gene corresponding to rPSC3 has been identified. Thus, mammaglobin and the cDNA encoding mammaglobin represent novel sequences heretofore unknown.

Using manual alignment with other sequences that had less significant BLAST scores with both mammaglobin and rPSC3 protein sequences, we identified other homologies with human clara cell 10kD protein (hCC10) (SEQ ID NO:8) (Peri et al, *J Clin Invest* 92:2099-2109, 1993 which is incorporated by reference) (FIG 3) and, in addition, with rabbit and mouse uteroglobin proteins (Miele et al., *Endocrine Rev 8:474-90*, 1987; Cato and Beato, *Anticancer Res* 5:65-72, 1985; Miele et al., *J Endocrinol Invest 17:*679-692, 1994 which are incorporated by reference). These homologies, depending on species, were 26% identity or 40% including conservative substitutions. In particular, a number of amino acids were perfectly conserved among all proteins, including Cys-3 and Cys-69 which are known to play a role in disulfide bond formation between uteroglobin subunits (see below). These homologies suggest that mammaglobin is a novel member of a small family of proteins that are secreted by epithelial cells (Miele et al, 1994, *supra*).

The hCC10 gene is the human homologue of rabbit and mouse uteroglobin genes (Peri et al, *J Clin Invest*

92:2099-2109, 1993 which is incorporated by reference). Uteroglobin was originally characterized as a secretory protein in rabbit uterus, but has since been found in other epithelial organs including lung, breast and prostate. Unlike rat prostatein, uteroglobin is a homodimeric protein coupled by two disulfide linkages at the conserved residues Cys-2 and Cys-69 (Miele et al, 1994, *supra*). Although uteroglobin gene transcription is regulated by steroid hormones, the ability of the protein itself to bind progesterone or other steroid hormones is controversial and again, the true biological function of this protein is unknown (Miele et al., 1994, *supra*).

Mammaglobin expression is restricted to the mammary gland. This is in contrast to the observation that rPSC3 is expressed in rat ventral prostate (Parker et al., *Ann N Y Acad Sci 438*:115-1124, 1984), and the expression of hCC10/uteroglobin in numerous tissues including lung, uterus, prostate, and breast (Miele et al., 1987, *supra*; Cato and Beato, *supra*; Miele et al., 1994 *supra*). Because of the sequence homology between mammaglobin and these proteins, we determined the pattern of tissue specific expression. The 500 bp mammaglobin message was easily detected in tumor specimen 2410 (the tissue from which this original sequence tag was isolated) and to a much less extent in normal human breast tissue (FIG 4A). The mammaglobin message could not be detected in the immortalized breast epithelial cell line B5-589. Expression of mammaglobin was also undetectable in human uterus and lung, two sites of uteroglobin expression.

Amplification using RT/PCR detected mammaglobin mRNA in both tumor 2410 and normal breast tissue, but not in 15 other tissues surveyed, including tissues that normally express rPSC3 and uteroglobin (lung, uterus, prostate), hormonally responsive and steroidogenic tissues (ovary, testis, placenta), and other secretory

epithelial organs (colon) (FIG 4B). Therefore, the expression of mammaglobin mRNA is relatively specific for mammary tissue.

Based on the studies in this report, mammaglobin is a relatively mammary-specific protein. Two other genes known to be overexpressed in breast carcinoma are erb-B and cyclin D (Jardines et al, *Pathobiology 61*:268-282, 1994; Keyomars and Pardee, *Proc Nat Acad Sci U.S. 90*:1112-1116, 1993 which is incorporated by reference). Unlike the overexpression of erb-B or cyclin D, the overexpression of mammaglobin may reflect a more specific alteration of the mammary epithelial cell rather than a general increased growth potential or mitotic rate. As such, appearance of mammaglobin gene dysregulation may have more specific import for the therapeutic vulnerability or clinical course of a tumor.

Mammaglobin expression could not be detected in normal lymph nodes or peripheral lymphocytes at the level of sensitivity afforded by a single step RT/PCR assay. This suggests that analysis of mammaglobin transcripts in peripheral lymph nodes may be useful for detecting occult breast cancer metastases, as has been suggested for other epithelial specific genes (Schoenfeld et al., *Cancer Res 54*:2986-90 which is incorporated by reference).

To demonstrate that the mammaglobin cDNA encoded a translatable protein, the cDNA clone was used in an *in vitro* translation assay. Figure 5 shows the protein product from a rabbit reticulocyte lysate programmed with the mammaglobin cDNA. An approximately 6 kD protein is generated using the mammaglobin cDNA. The apparent molecular weight is smaller than that predicted from conceptual translation of the open reading frame, but this finding is commonly observed with rabbit and human uteroglobin translation products as well.

Although we detected overexpression of mammaglobin RNA in one tumor specimen (i.e. 2410), it was not clear

at what frequency this overexpression is seen in other breast carcinomas. We therefore examined a panel of fifteen, stage I primary breast carcinomas of differing histological types by Northern blot hybridization with the mammaglobin cDNA probe. Because of potential variability in expression due to environment influences (e.g. patient hormonal status), we also sought to compare tumor specimens directly with patient-matched normal breast tissues samples, although this was not possible in many cases. As shown in FIG 6, the 500 bp mammaglobin mRNA was again detected in normal breast tissue and tumor 2410. Mammaglobin was also detected in three other tumors, two of which demonstrated little or no expression in patient-matched normal tissue. In all, 4 of 15 (27%) of tumors examined overexpressed mammaglobin mRNA. These data suggest that overexpression of mammaglobin is not unique to a single tumor specimen and is, in fact, relatively frequent among primary breast tumors. Furthermore, the fact that all tumors examined were stage I suggests that this dysregulation occurs relatively early in the progression of breast neoplasia.

Because Applicants believe mammaglobin is likely to be a secreted protein, its presence would be expected to be detectable in sera from patients whose tumor overexpresses this gene product. As such, mammaglobin is likely to be as clinically useful as prostate specific antigen (PSA) and other solid tumor markers for managing patients with breast cancer (Tumor markers in diagnostic pathology, *Clin Lab Med 10*:1-250, 1990 which is incorporated by reference).

We determined the prevalence of mammaglobin as a tumor marker in the general population of breast cancer tumors by examining the expression of mammaglobin in several primary breast carcinomas. Although the number of sp cimens examined in this study was small, 27% of tumors evaluated overexpressed mammaglobin mRNA. This

percentage is comparable to the prevalence of other genetic alterations such as erb-B amplification and p53 mutation (Slamon et al. *Sci 244*:707-712, 1989; Thor et al, *J Nat'l Cancer Inst 84*:845-855, 1992 which are incorporated by reference). Furthermore, because we have restricted our analysis to stage I tumors, overexpression of mammaglobin would actually be more prevalent than any other genetic alteration reported in this subgroup of tumors (Alllerd et al, *J Nat'l Cancer Inst 85*:200-206, 1993 which is incorporated by reference).

The identification of mammaglobin as a breast cancer marker provides the basis for another aspect of the present invention, which involves methods for detecting the presence of breast cancer in a patient. The term "detection" as used herein in the context of detection of breast neoplastic disease is intended to be a comprising aspect of the determining of the presence of breast cancer in a patient, the distinguishing of breast cancer from other diseases, the estimation of prognosis in terms of probable outcome of the disease and prospect for recovery, the monitoring of the disease status or the recurrence of the disease, the determining of a preferred therapeutic regimen for the patient and the targeting of antitumor therapy.

The method for detecting breast cancer comprises hybridizing a polynucleotide to mRNA from breast neoplasia cells. The polynucleotide comprises SEQ ID NO:1 or a derivative of SEQ ID NO:1. By derived from a nucleotide sequence it is meant that the derived nucleotide sequence is substantially the same as the sequence from which it is derived in that the derived nucleotide sequence has sufficient sequence complementarily to the sequence from which it is derived to hybridize to mRNA from breast neoplasia cells under

the same stringency conditions that the sequence from which it is derived hybridizes to the mRNA from breast neoplasia cells.

The derived nucleotide sequence is not necessarily physically derived from the nucleotide sequence, but may be generated in any manner including for example, chemical synthesis or DNA replication or reverse transcription or transcription.

To detect the presence of mRNA encoding mammaglobin in a detection system for breast cancer, a sample is obtained from a patient. The sample can be a tissue biopsy sample or a sample of blood, plasma, serum or the like. The sample may be treated to extract the nucleic acids contained therein. The resulting nucleic acid from the sample is subjected to gel electrophoresis or other size separation techniques.

Detection involves contacting the nucleic acids and in particular the mRNA of the sample with a DNA sequence serving as a probe to form hybrid duplexes. The term "probe" refers to a structure comprised of a polynucleotide which forms a hybrid structure with a target sequence, due to complementarily of probe sequence with a sequence in the target region.

Detection of the resulting duplex is usually accomplished by the use of labeled probes. Alternatively, the probe may be unlabeled, but may be detectable by specific binding with a ligand which is labeled, either directly or indirectly. Suitable labels and methods for labeling probes and ligands are known in the art, and include, for example, radioactive labels which may be incorporated by known methods (e.g., nick translation or kinasing), biotin, fluorescent groups, chemiluminescent groups (e.g., dioxetanes, particularly triggered dioxetanes), enzymes, antibodies, and the like.

When using the cDNA encoding mammaloglobin or a derivative thereof as a probe, high stringency conditions

can be used in order to prevent false positives. When using sequences derived from mammaglobin, less stringent conditions can be used. The stringency of hybridization is determined by a number of factors during hybridization and during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. These factors are outlined in, for example, Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2d ed., 1989).

In order to increase the sensitivity of the detection in a sample of mRNA encoding mammaglobin, the technique of reverse transcription/polymerization chain reaction (RT/PCR) can be used to amplify cDNA transcribed from mRNA encoding mammaglobin. The method of RT/PCR is well known in the art (for example, see Watson and Fleming, *supra*).

The RT/PCR method can be performed as follows. Total cellular RNA is isolated by, for example, the standard guanidium isothiocyanate method and the total RNA is reverse transcribed. The reverse transcription method involves synthesis of DNA on a template of RNA using a reverse transcriptase enzyme and a 3' end primer. Typically, the primer contains an oligo(dT) sequence. The cDNA thus produced is then amplified using the PCR method and mammaglobin specific primers. (Belyavsky et al, *Nucl Acid Res* 17:2919-2932, 1989; Krug and Berger, *Methods in Enzymology*, Academic Press, N.Y., Vol.152, pp. 316-325, 1987 which are incorporated by reference)

The polymerase chain reaction method is performed using two oligonucleotide primers that are complementary to the two flanking regions of the DNA segment to be amplified. The upstream and down stream primers are typically from 20 to 30 base pairs in length and hybridize to the flanking regions for replication of the nucleotide sequence. The polymerization is catalyzed by a DNA-polymerase in the presence of deoxynucleotide

triphosphates or nucleotide analogs to produce doubl - stranded DNA molecules. The double strands are then separated by any denaturing method including physical, chemical or enzymatic. Commonly, the method of physical denaturation is used involving heating the nucleic acid, typically to temperatures from about 80°C to 105°C for times ranging from about 1 to 10 minutes. The process is repeated for the desired number of cycles.

The primers are selected to be substantially complementary to the strand of cDNA being amplified. Therefore, the primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with the strand being amplified.

Following amplification, the PCR product is then detected by ethidium bromide staining (Sambrook, et al., 1989, *supra*).

In another embodiment of the present invention, the mammaglobin cDNA sequence or derivative thereof can be used to characterize any alteration of the mammaglobin gene (i.e. gene rearrangement, gene amplification, or gene deletion) in a specimen from a breast-cancer patient. This provides a method whereby patient specimens or samples, which do not contain intact mRNA, can still be examined for changes in gene structure.

In one application of this technique, the mammaglobin cDNA sequence or derivative thereof is hybridized to patient genomic DNA that had been isolated from a patient's tumor, normal tissue, or lymphocytes and digested with one or more restriction endonucleases. Using the Southern blot protocol, which is well known in the art, this assay determines whether a patient or a patient's breast tumor has a mammaglobin gene, which was deleted, rearranged, or amplified. Detection of these changes can then provide important information useful for predicting prognosis and for patient management.

In a second application of this technique, one or more pairs of oligonucleotide primers based on the mammaglobin cDNA sequence or derivative thereof could be used in the polymerase chain reaction to amplify segments of the mammaglobin gene from a patient sample. Analysis of the resulting PCR products indicate whether a particular segment of the mammaglobin gene is deleted or rearranged. Such information is useful for prognosis and patient management.

The present invention further provides for methods to detect the presence of the polypeptide, mammaglobin, in a sample obtained from a patient. Any method known in the art for detecting proteins can be used. Such methods include, but are not limited to immunodiffusion, immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical techniques, agglutination and complement assays. (for example see *Basic and Clinical Immunology*, Sites and Terr, eds., Appleton & Lange, Norwalk, Conn. pp 217-262, 1991 which is incorporated by reference). Preferred are binder-ligand immunoassay methods including reacting antibodies with an epitope or epitopes of mammaglobin and competitively displacing a labeled mammaglobin protein or derivative thereof.

As used herein, a derivative of mammaglobin is intended to refer to a polypeptide containing amino acids or modified amino acids in which the polypeptide derivative cross-reacts with mammaglobin. By cross-reaction it is meant that an antibody reacts with an antigen other than the one that induced its formation.

Numerous competitive and non-competitive protein binding immunoassays are well known in the art. Antibodies employed in such assays may be unlabeled, for example as used in agglutination tests, or labeled for us a wide vari ty of assay methods. Labels that can b used include radionuclides, enzymes, fluorescers,

chemiluminescers, enzyme substrates or co-factors, enzyme inhibitors, particles, dyes and the like for use in radioimmunoassay (RIA), enzyme immunoassays, e.g., enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassays and the like.

Polyclonal or monoclonal antibodies to mammaglobin or an epitope thereof can be made for use in immunoassays by any of a number of methods known in the art. By epitope reference is made to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids in a spacial conformation which is unique to the epitope. Generally an epitope consists of at least 5 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2 dimensional nuclear magnetic resonance.

One approach for preparing antibodies to a protein is the selection and preparation of an amino acid sequence of all or part of the protein, chemically synthesizing the sequence and injecting it into an appropriate animal, usually a rabbit or a mouse.

Methods for preparation of mammaglobin or an epitope thereof include, but are not limited to chemical synthesis, recombinant DNA techniques or isolation from biological samples. Chemical synthesis of a peptide can be performed, for example, by the classical Merrifeld method of solid phase peptide synthesis (Merrifeld, *J Am Chem Soc 85:*2149, 1963 which is incorporated by reference) or the FMOC strategy on a Rapid Automated Multiple Peptide Synthesis system (DuPont Company, Wilmington, DE) (Caprino and Han, *J Org Chem 37*:3404, 1972 which is incorporated by reference).

Polyclonal antibodies can be prepared by immunizing rabbits by injecting antigen into the popliteal lymph nodes followed by subsequent boosts at two week intervals with intraperitoneal injection of

antigen. The animals are bled and sera assayed against purified mammaglobin protein usually by ELISA. Monoclonal antibodies can be prepared after the method of Milstein and Kohler by fusing splenocytes from immunized mice with continuously replicating tumor cells such as myeloma or lymphoma cells. (Milstein and Kohler *Nature 256*:495-497, 1975; Gulfre and Milstein, *Methods in Enzymology: Immunochemical Techniques* 73:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). The hybridoma cells so formed are then cloned by limiting dilution methods and supernates assayed for antibody production by ELISA or RIA.

The unique ability of antibodies to recognize and specifically bind to target antigens expressed by a tumor cell provides an approach for the treatment of cancer. (For review see LoBuglio and Saleh, *Am J Med Sci 304*:214-224, 1992; Bagshawe, *Adv Pharmacol 24*:99-121, 1993 which are incorporated by reference). Thus, another aspect of the present invention provides for a method for preventing the onset and treating breast cancer in an animal based upon the use of antibodies to mammaglobin, which has been discovered to be overexpressed by breast cancer cells. Specific antibodies to mammaglobin, either polyclonal or monoclonal, are produced by any method known in the art. For example, murine or human monoclonal antibodies can be produced by hybridoma technology. Alternatively, mammaglobin, or an immunologically active fragment thereof, or an anti-idiotypic antibody, or fragment thereof can be administered to an animal to elicit the production of antibodies capable of recognizing the mammaglobin-expressing cells.

The antibodies so produced or fragments thereof ar labeled with one or more oncolytic substances such as radionuclides, toxins, or cytotoxic drugs and

administered to a patient suspected of having breast cancer. The binding of the labeled antibody to the mammaglobin being overexpressed by the breast cancer cell will cause the death of the cancer cell.

Any of a variety of oncolytic substances known in the art can be used to produce such labeled antibodies. For example, immunotoxins can be made by coupling plant and bacterial toxins to antibodies. Such toxins include, for example, ricin, diphtheria toxin and *Pseudomonas* exotoxin A. Drug-antibody conjugates can also be made in which chemotherapeutic agents are linked to the antibody. Chemotherapeutic agents suitable for such use include, for example, tomoxifen, doxorubicin, methotrexate, chlorambucil, *Vinca* alkaloids, and mitomycin. In addition, radioimmunoconjugates can be made in which a radionuclide is stably linked to the antibody. Radionuclides suitable for making radioimmunoconjugates include, for example, ß-emmitters such as ^{131}I, ^{188}Re, ^{186}Re, ^{67}Cu, ^{90}Y and ^{47}Sc; α-emitters such as ^{211}At, ^{212}Bi and ^{212}Pb; auger electron emitters such as ^{125}I and ^{77}Br; and fissionable nuclides such as ^{10}B.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

In the examples below, cell lines were obtained from American Type Culture Collection and grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Tissue biopsy specimens were obtained

from the Human Cooperative Tissue Network (LiVolsi et al, *Cancer 71*:1391-1394, 1993 which is incorporated by reference).

Example 1

This example illustrates the isolation of mammaglobin cDNA.

Total cellular RNA from the cell line MDA-MB415 was isolated using the standard guanidinium isothiocyanate method. (Belyavsky et al, *supra*). This RNA was used in the RACE PCR procedure employing the Amplifinder kit (Clonetech) and following the manufacturer's protocol.

The synthesis of first strand cDNA was performed in a standard reaction containing 1 µg RNA, 10 µM specific mammaglobin primer D2R (5'-ATA AGA AAG AGA AGG TGT GG-3')(SEQ ID NO:4), 4µl of 5X RT buffer (250 mM TrisCl pH8.3, 375mM Kcl, 15mM $MgCl_2$), 2 µl of 100mM DTT, 1 µl of 10 mM dNTPs and 200 units of Superscript™ II reverse transciptase (Gibco/BRL) in a reaction volume 20 µl. The reaction proceeded for 1 hour at 45°C and was terminated by incubating at 95°C for 5 minutes. RNA was hydrolyzed with 400 µM NaOH at 65°C for 30 minutes and neutralized with 400 µM acetic acid. Reaction was then added to 3 volumes of 6M NaI and 10 µl of treated glass beads. Beads were washed three times with 80% EtOH and nucleic acid was eluted from the beads in 45 µl of water. Nucleic acid was then precipitated and resuspended in 10 µl of water. The purified first strand cDNA was ligated to the manufacturer's provided anchor oligonucleotide (SEQ ID NO:9, 5'-CAC GAA TTC ACT ATC GAT TCT GGA ACC TTC AGA GG-3'), using T4 RNA ligase at 27° for 20 hours. One tenth of a ligation reaction was used for PCR amplification in a 50 µl reaction containing 1 µM manufacturer's anchor primer (SEQ ID NO:10, 5'-CTG GTT CGG CCC ACC TCT GAA GGT TCC AGA ATC GAT AG-3'), 1 µM

mammaglobin specific primer D2Rb (SEQ ID NO:11, 5'-AAT CCG TAG TTG GTT TCT CAC C-3'), 200 µM dNTPs, 5 units of Vent™ DNA polymerase, and 1X polymerase buffer (10mM Kcl, 20 mM TrisCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100). The reaction was incubated at 94° for 2 minutes and then 94° for 45 seconds, 50° for 1 minute, and 72° for 90 seconds for a total of 40 times.

The two downstream mammaglobin-specific nested oligonucleotides were D2R (SEQ ID NO:4) and D2Rb (SEQ ID NO:11). An upstream mammaglobin-specific control oligonucleotide was also used as per the manufacturer's recommendations, D2F (5'-CTT TCT GCA AGA CCT TTG GC-3') (SEQ ID NO:12). All PCR amplifications were performed with Vent DNA polymerase (New England Biolabs). The amplified RACE product was digested with EcoRI and ligated into the EcoRI and SmaI sites of the plasmid vector pGEM7Z (Promega).

All sequencing was performed using the Taq DNA polymerase thermal cycle sequencing kit as per the manufacture's protocol (Promega). Briefly the procedure used is as follows.

10 pmol of sequence specific oligonucleotide was end labeled with 10 pmol of ^{32}P-γ ATP (3,000 Ci/mmol and 10 mCi/ml) using T4 polynucleotide kinase in a 10 µl reaction for 30 minutes at 37°C. A polymerization reaction containing 100 ng of plasmid template, 1.5 pmol of labeled sequencing primer, and 5 units of sequencing grade Taq polymerase was created in 17 µl of the manufacturer's provided sequencing buffer. This reaction was aliquoted to a set of four reaction tubes containing manufacturer's provided mix of deoxynucleotides and either dideoxy-A, C, G, or T. The set of four tubes were incubated at 95°C for 2 minutes and then, 94°C for 45 seconds, 45°C for 30 seconds, and 72°C for 1 minute for 30 times. After reactions were completed, 3 µl of 80% formamide/bromphenol blue dye was added to each tube.

Samples were heated to 70°C for 2 minutes and loaded on a 6% acrylamide/7.5 M urea sequencing gel and run for 2-4 hours and 60 W constant power. The gel was dried and then exposed to Kodak XAR5 Xray film for 2 to 24 hours.

The sequence thus obtained was a 403 bp fragment (SEQ ID NO:5) as shown in FIG 2, solid bar. In earlier work the DEST002 Tag sequence was isolated (Watson and Fleming, *supra*). This sequence was a 206 bp fragment (SEQ ID NO:6) as shown in FIG 2, open bar. Combining the information from these two sequences allowed the full-length 503 bp cDNA of mammaglobin to be deduced. (FIG 2).

Example 2

This example demonstrates that mammaglobin expression is restricted to mammary gland tumor cells and to a lesser extent normal mammary gland cells.

Total cellular RNA samples were isolated using the standard guanidinium isothiocyanate method and treated with RNase-free DNase (Promega). For RT/PCR analysis, 1 µg of indicated total RNA was reverse transcribed with oligo dT_{21} (SEQ ID NO:13) and Superscript II reverse transcriptase (Gibco/BRL) according to the manufacture's protocol.

Two hundred ng of oligo dT_{21} (SEQ ID NO:13) and 1 µg of total RNA were incubated at 65°C for 5 minutes in a 10 µl volume. Sample was chilled on ice and added to it were 4µl of 5X RT buffer (250 mM TrisCl pH8.3, 375 mM Kcl, 15 mM $MgCl_2$), 2 µl of 100mM DTT, 1 µl of 10mM dNTPs and 200 units of Superscript™ II reverse transcriptase (Gibco/BRL). The reaction proceeded for 1 hour at 45°C and was terminated by incubating at 95°C for 5 minutes.

One tenth of each RT reaction was subject to PCR analysis using the mammaglobin specific primers D2R (5'-ATA AGA AAG AGA AGG TGT GG-3') (SEQ ID NO:4) and d2102

(5'-CAG CGG CTT CCT TGA TCC TTG-3') (SEQ ID NO:3) and standard reaction conditions for 40 cycles at 94° x 30 sec./55° x 1 min./72° x 1 min.

For Northern analysis, 20 µg of total RNA was analyzed as previously described (Watson and Fleming, *supra*) using the full length mammaglobin cDNA probe. Integrity and equal loading of each RNA sample was assessed by ethidium bromide staining.

As shown in FIG 4A, the 500 bp mammaglobin message is easily detected in tumor specimen 2410 (the tissue from which this original DEST was isolated) and to a much less extent in normal human breast tissue but not in the immortalized breast epithelial cell line B5-589, or in human lung, placenta, uterus and ovary (FIG 4A). Following amplification using RT/PCR analysis, mammaglobin expression was still not detected in 15 tissues surveyed (FIG 4B). Detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) message (FIG 4B) and EGF receptor message (data not shown) in these reactions demonstrated that absence of expression was not due to degraded RNA or other trivial explanations. Thus the expression of mammaglobin mRNA is relatively specific for mammary tissue.

Example 3

This example demonstrates that the mammaglobin cDNA encodes a translatable nucleotide sequence which results in protein product of appropriately predicted molecular mass. *In vitro* translations were performed using the TNT™ rabbit reticulocyte translation kit with T7 RNA polymerase (Promega) and ^{35}S-Methionine (>1000 Ci/mmol; 10 mCi/ml, Amersham) according to the manufacturer's protocol.

To 25 µl of TNT™ rabbit reticulocyte lystae was added 2 µl of manufacturer's prepared reaction buffer, T7 RNA polymerase, 20µM amino acid mixture minus methionin , 40µCi^{35}S-methionine (1,000 Ci/mmol and 10 mCi/ml), 40

units ribonuclease inhibitor, 1 µg of mammaglobin/pGEM7 plasmid, and sufficient DEPC treated water to create a final reaction volume of 50 µl. This reaction was incubated at 30°C for 60 minutes. 5µl of this reaction was removed into 20µl of SDS gel buffer, boiled for 2 minutes, and loaded on a 17.5% SDS-polyacrylamide gel.

Rabbit reticulocyte lysate programmed with mammaglobin cDNA produced a 6kD protein while that programmed with no cDNA did not produce any protein product.

Example 4

This example illustrates the prevalence of overexpression of mammaglobin in primary breast carcinoma.

To determine the frequency of mammaglobin overexpression in breast carcinomas, we examined a panel of fifteen, stage I primary breast carcinomas of differing histological types using Northern blot hybridization with the mammaglobin cDNA probe. Patient-matched normal breast tissues samples were also compared in tissues from two patients (FIG 6). The 500 bp mammaglobin mRNA was detected in normal breast tissue and tumor 2410 and in three other tumors, two of which when tested demonstrated little or no expression in patient-matched normal tissue (B015 v. B016; B022 v. B023) (FIG 6). In all, 4 of 15 (27%) of tumors examined overexpressed mammaglobin mRNA. These data indicate that overexpression of mammaglobin is not unique to a single tumor specimen and is, in fact, relatively frequent among primary breast tumors. Furthermore, the fact that all tumors examined were stage I suggests that this dysregulation occurs relatively early in the progression of breast neoplasia.

EXAMPLE 5

The following example illustrates the detection of mammaglobin protein using polyclonal antibody.

Polyclonal antibody was prepared by coupling a peptide corresponding to the 16 C-terminal amino acids predicted from mammaglobin cDNA (Glu-Val-Phe-Met-Gln-Leu-Ile-Tyr-Asp-Ser-Ser-Leu-Cys-Asp-Leu-Phe, SEQ ID NO:14) to Keyhole Lymphet Hemocyanin and injecting into rabbits with Freund's adjuvant. The inoculated rabbits were boosted at three week intervals and on week 12, the rabbits were bled and the sera was assayed for its ability to detect mammaglobin. Serum-free conditioned medium was harvested from the breast tumor cell lines MDA-MB-415 and MCF-7 (24 hour collections). MDA-MB-415 had been identified earlier as a cell line that overexpresses the mammaglobin message and MCF-7 had been identified as a cell line that produces no detectable mammaglobin. The conditioned media was resolved on a 12% SDS acrylamide gel under reducing conditions, blotted onto a Nytran filter, and analyzed by standard Western blot protocols using the described antibody to the C-terminal peptide as the primary antibody in this assay. After primary antibody binding, the blot was washed and secondary antibody (goat anti-rabbit) was added. Mammaglobin-antibody complexes were visualized by enzyme-linked chemoluminescence (ECL Western Blotting Detecting Reagent, Amersham, Arlington Heights, IL). The conditioned media for the MDA-MB-415 cell line showed a mammaglobin band of apparent molecular weight of 20 kd and this band was not detected in the conditioned medium of the MCF-7 cell line. Thus, MDA-MB-415 cells secrete mammaglobin protein but MCF-7 cells do not.

To further illustrate the specificity of this protein, the conditioned media and cell lysate of the MDA-MB-415 cell line were assayed by Western blot analysis, with the antibody to the C-terminal peptide, in

the presence and absence of the competing peptide used to generate the antibody. Visualization of mammaglobin-antibody complexes were as discussed above. As seen in Figure 7, in the absence of competing peptide (-), the conditioned media (S) has the 20 kd band representative of the mammaglobin protein. The cell lysate (C) showed several bands at 14 kd, 20 kd, and higher molecular weight. The 14 kd band likely represents mammaglobin in the unprocessed form. The cDNA for mammaglobin has a consensus N-glycosylation site and the observed, secreted 20 kd form likely represents some processed form of the protein. When the Western blot is performed in the presence of the competing peptide (+), the secreted form and intracellular forms of mammaglobin are not visualized, indicating that these proteins contain the peptide to which the antibody was synthesized.

This antibody to the C-terminal peptide has also detected similar bands in cell lysates from primary breast tumor specimens (Fig. 8). In addition, the antibody showed reactivity to breast tumor cells by immunohistochemical staining of paraffin-fixed sections of breast cancer obtained from a patient specimen (Fig. 9). The immunohistochemical staining was performed using the antibody to the mammaglobin peptide and goat anti-rabbit antibody tagged with horseradish peroxidase and 3, 3' diamino benzene tetrahydrochloride (DAB) as substrate. Cells expressing the mammaglobin protein showed a brown staining.

From these results, we believe that mammaglobin is a secreted protein, that the mammaglobin protein is synthesized as a precursor protein and post-translational modifications increase its apparent molecular weight necessary prior to secretion; and that the mammaglobin protein can be detected in human breast tumor specimens. The detection of a mammaglobin protein is applicable in cancer diagnostics using the mammaglobin protein as a

breast tumor marker, in assessing breast tumor relapse, in monitoring autologous bone marrow/stem cell transplants for contaminating tumor cells, in breast tumor vaccines, and in targeting breast tumor cells for therapeutic intervention via antibody-mediated complexes.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: WATSON, MARK A.
FLEMING, TIMOTHY P.

(ii) TITLE OF INVENTION: DNA SEQUENCE AND ENCODED MAMMARY-SPECIFIC BREAST CANCER PROTEIN

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ROGERS, HOWELL & HAFERKAMP
(B) STREET: 7733 FORSYTH BOULEVARD, SUITE 1400
(C) CITY: ST. LOUIS
(D) STATE: MISSOURI
(E) COUNTRY: USA
(F) ZIP: 63105-1817

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HOLLAND, DONALD R.
(B) REGISTRATION NUMBER: 35,197
(C) REFERENCE/DOCKET NUMBER: 964796

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (314) 727-5188
(B) TELEFAX: (314) 727-6092

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 503 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GACAGCGGCT TCCTTGATCC TTGCCACCCG CGACTGAACA CCGACAGCAG CAGCCTCACC 60

ATGAAGTTGC TGATGGTCCT CATGCTGGCG GCCCTCTCCC AGCACTGCTA CGCAGGCTCT 120

GGCTGCCCCT TATTGGAGAA TGTGATTTCC AAGACAATCA ATCCACAAGT GTCTAAGACT 180

GAATACAAAG AACTTCTTCA AGAGTTCATA GACGACAATG CCACTACAAA TGCCATAGAT 240

GAATTGAAGG AATGTTTTCT TAACCAAACG GATGAAACTC TGAGCAATGT TGAGGTGTTT 300

ATGCAATTAA TATATGACAG CAGTCTTTGT GATTTATTTT AACTTTCTGC AAGACCTTTG 360

GCTCACAGAA CTGCAGGGTA TGGTGAGAAA CCAACTACGG ATTGCTGCAA ACCACACCTT 420

CTCTTTCTTA TGTCTTTTTA CTACAAACTA CAAGACAATT GTTGAAACCT GCTATACATG 480

TTTATTTTAA TAAATTGATG GCA 503

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Leu Leu Met Val Leu Met Leu Ala Ala Leu Ser Gln His Cys
1 5 10 15

Tyr Ala Gly Ser Gly Cys Pro Leu Leu Glu Asn Val Ile Ser Lys Thr
20 25 30

Ile Asn Pro Gln Val Ser Lys Thr Glu Tyr Lys Glu Leu Leu Gln Glu
35 40 45

Phe Ile Asp Asp Asn Ala Thr Thr Asn Ala Ile Asp Glu Leu Lys Glu
50 55 60

Cys Phe Leu Asn Gln Thr Asp Glu Thr Leu Ser Asn Val Glu Val Phe
65 70 75 80

Met Gln Leu Ile Tyr Asp Ser Ser Leu Cys Asp Leu Phe
85 90

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGCGGCTTC CTTGATCCTT G 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATAAGAAAGA GAAGGTGTGG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 403 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACAGCGGCT TCCTTGATCC TTGCCACCCG CGACTGAACA CCGACAGCAG CAGCCTCACC 60

ATGAAGTTGC TGATGGTCCT CATGCTGGCG GCCCTCTCCC AGCACTGCTA CGCAGGCTCT 120

GGCTGCCCCT TATTGGAGAA TGTGATTTCC AAGACAATCA ATCCACAAGT GTCTAAGACT 180

GAATACAAAG AACTTCTTCA AGAGTTCATA GACGACAATG CCACTACAAA TGCCATAGAT 240

GAATTGAAGG AATGTTTTCT TAACCAAACG GATGAAACTC TGAGCAATGT TGAGGTGTTT 300

ATGCAATTAA TATATGACAG CAGTCTTTGT GATTTATTTT AACTTTCTGC AAGACCTTTG 360

GCTCACAGAA CTGCAGGGTA TGGTGAGAAA CCAACTACGG ATT 403

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 206 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTATGCAAT TAATATATGA CAGCAGTCTT TGTGATTTAT TTTAACTTTC TGCAAGACCT 60

TTGGCTCACA GAACTGCAGG GTATGGTGAG AAACCAACTA CGGATTGCTG CAAACCACAC 120

CTTCTCTTTC TTATGTCTTT TTACTACAAA CTACAAGACA ATTGTTGAAA CCTGCTATAC 180

ATGTTTATTT TAATAAATTG ATGGCA 206

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Lys Leu Val Phe Leu Phe Leu Leu Val Thr Ile Pro Ile Cys Cys
1 5 10 15

Tyr Ala Ser Gly Ser Gly Cys Ser Ile Leu Asp Glu Val Ile Arg Gly
20 25 30

Thr Ile Asn Ser Thr Val Thr Leu His Asp Tyr Met Lys Leu Val Lys
35 40 45

Pro Tyr Val Gln Asp His Phe Thr Glu Lys Ala Val Lys Gln Phe Lys
50 55 60

Gln Cys Phe Leu Asp Gln Thr Asp Lys Thr Leu Glu Asn Val Gly Val
65 70 75 80

Met Met Glu Ala Ile Phe Asn Ser Glu Ser Cys Gln Gln Pro Ser
85 90 95

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Leu Ala Val Thr Leu Thr Leu Val Thr Leu Ala Leu Cys Cys
1 5 10 15

Ser Ser Ala Ser Ala Glu Ile Cys Pro Ser Phe Gln Arg Val Ile Glu
20 25 30

Thr Leu Leu Met Asp Thr Pro Ser Ser Tyr Glu Ala Ala Met Glu Leu
35 40 45

Phe Ser Pro Asp Gln Asp Met Arg Glu Ala Gly Ala Gln Leu Lys Lys
50 55 60

Leu Val Asp Thr Leu Pro Gln Lys Pro Arg Glu Ser Ile Ile Lys Leu
65 70 75 80

Met Glu Lys Ile Ala Gln Ser Ser Leu Cys Asn
85 90

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CACGAATTCA CTATCGATTC TGGAACCTTC AGAGG 35

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTGGTTCGGC CCACCTCTGA AGGTTCCAGA ATCGATAG 38

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATCCGTAGT TGGTTTCTCA CC 22

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTTTCTGCAA GACCTTTGGC 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTTTTTTT TTTTTTTTTT T 21

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Glu	Val	Phe	Met	Gln	Leu	Ile	Tyr	Asp	Ser	Ser	Leu	Cys	Asp	Leu	Phe
1				5				10					15		

What is Claimed is:

1. A purified and isolated polynucleotide containing SEQ ID NO:1 or a derivative thereof.

2. The purified and isolated polynucleotide of claim 1 wherein the polynucleotide is SEQ ID NO:1 or a derivative thereof.

3. The purified and isolated polynucleotide according to claim 1 wherein the polynucleotide contains a nucleotide sequence that encodes for expression, a peptide having the sequence of SEQ ID NO:2.

4. A purified and isolated peptide containing SEQ ID NO:2 or a derivative thereof.

5. The purified and isolated peptide according to claim 4 wherein the peptide is SEQ ID NO:2 or a derivative thereof.

6. A method for detecting the presence of breast cancer in a patient comprising detecting and/or quantitating the presence of mRNA encoding the peptide of SEQ ID NO:2 or a derivative thereof in a sample from the patient wherein an elevated concentration of said mRNA above the concentration for a healthy individual indicates the presence of breast cancer cells.

7. The method according to claim 6 wherein the detecting and/or quantitating step further comprises the steps of:

(a) providing a polynucleotide containing SEQ ID NO:1 or a derivative thereof,

(b) incubating the polynucleotide with the sample under conditions in which the polynucleotide can hybridize with mRNA from breast neoplasia cells, and

(c) detecting the existence of a DNA-RNA hybridization complex.

8. The method according to claim 7 wherein the polynucleotide is SEQ ID NO:1.

9. The method according to claim 7 wherein the polynucleotide contains a nucleotide sequence that

encodes for expression, a peptide having the sequence of SEQ ID NO:2.

10. A kit for detection of the presence of breast neoplasia cells in a sample comprising a polynucleotide containing SEQ ID NO:1 or a derivative thereof packaged in a container.

11. The kit according to claim 10 wherein the polynucleotide is SEQ ID NO:1.

12. The kit according to claim 10 wherein the polynucleotide contains a nucleotide sequence that encodes for expression, a peptide having the sequence of SEQ ID NO:2.

13. The method according to claim 6 wherein the detecting and/or quantitating step further comprises the steps of:

(a) producing a cDNA from mRNA using the reverse transcription method in a sample obtained from a patient,

(b) providing two oligonucleotides which are primers for the polymerase chain reaction method and which flank or lie within a cDNA encoding SEQ ID NO:2,

(c) amplifying the cDNA encoding mammaglobin by the polymerase chain reaction method, and

(d) detecting the presence of the cDNA encoding SEQ ID NO:2.

14. The method according to claim 13 wherein one of said two oligonucleotides comprises SEQ ID NO:3 and the other comprises SEQ ID NO:4.

15. A kit for detection of the presence of breast neoplasia cells in a sample comprising two oligonucleotides which are primers for the polymerase chain reaction method and which flank or lie within a cDNA encoding SEQ ID NO:2 packaged in a container.

16. The kit according to claim 15 wherein one of said two oligonucleotides comprises SEQ ID NO:3 and the other comprises SEQ ID NO:4.

17. A method for detecting the presence of breast cancer in a patient comprising detecting and/or quantitating the presence of a protein containing SEQ ID NO:2 in a sample from the patient wherein an elevated concentration of said protein above the concentration for a healthy individual indicates the presence of breast cancer.

18. The method according to claim 17 wherein the detecting and/or quantitating step further comprises reacting a purified antibody with the protein containing SEQ ID NO:2 or an epitope thereof produced by a breast cancer cell and detecting a binding of the protein containing SEQ ID NO:2 or an epitope thereof with the antibody.

19. The method according to claim 18 wherein the purified antibody is a polyclonal antibody.

20. The method according to claim 19 wherein the purified antibody is a monoclonal antibody.

21. A kit for detection of the presence of breast cancer cells in a sample comprising a purified antibody that is capable of detectably reacting with a protein containing SEQ ID NO:2 or an epitope thereof produced by a breast cancer cell packaged in a container.

22. The kit according to claim 21 wherein the purified antibody is a polyclonal antibody.

23. The kit according to claim 22 wherein the purified antibody is a monoclonal antibody.

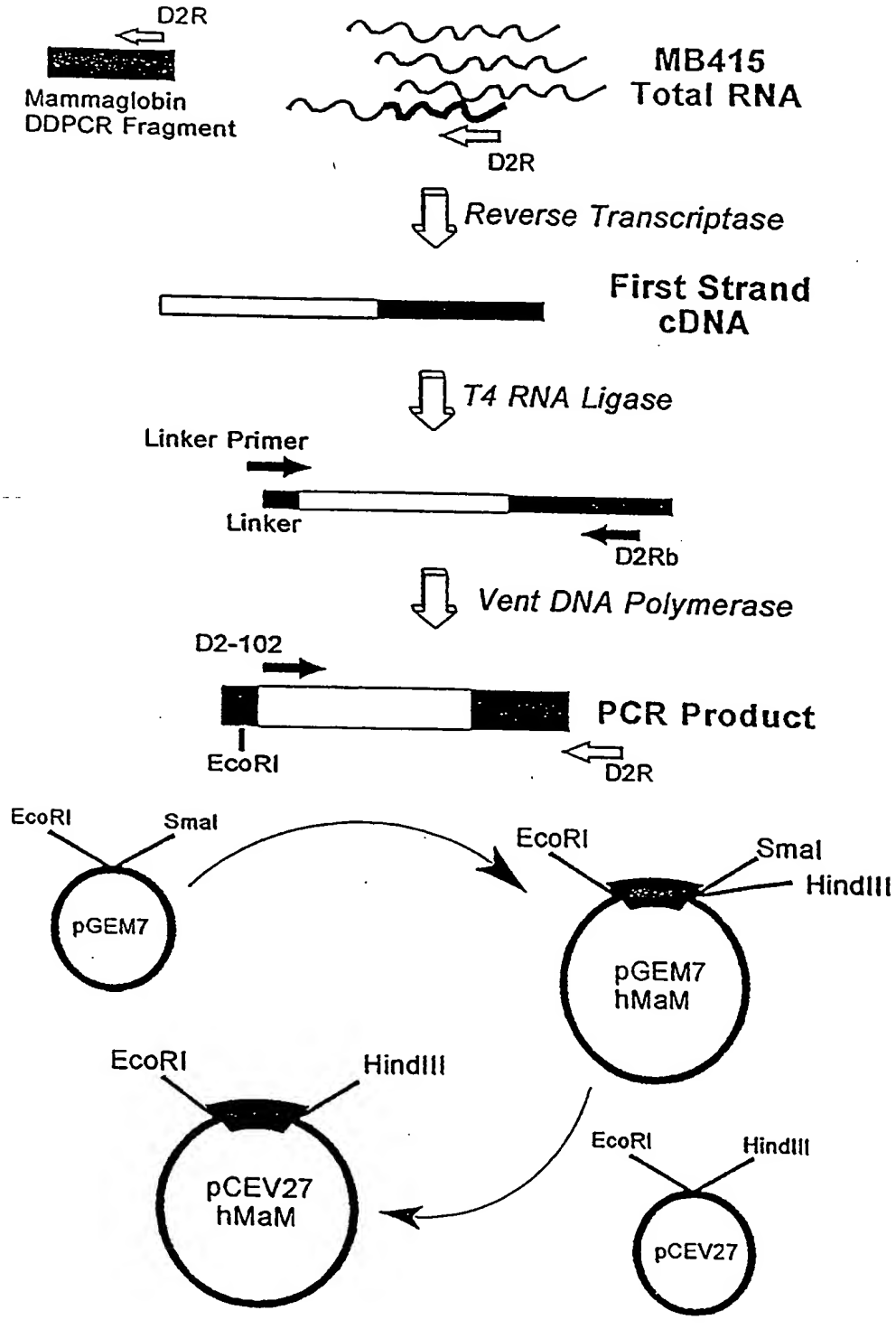
D2R
Mammaglobin
DDPCR Fragment
MB415
Total RNA
D2R
Reverse Transcriptase
First Strand
cDNA
T4 RNA Ligase
Linker Primer
Linker
D2Rb
Vent DNA Polymerase
D2-102
PCR Product
EcoRI
D2R
EcoRI
SmaI
pGEM7
EcoRI
SmaI
HindIII
pGEM7
hMaM
EcoRI
HindIII
pCEV27
hMaM
EcoRI
HindIII
pCEV27

Figure 1

```
          9          18          27          36          45          54
5' GAC AGC GGC TTC CTT GAT CCT TGC CAC CCG CGA CTG AAC ACC GAC AGC AGC AGC

         63          72          81          90          99         108
CTC ACC ATG AAG TTG CTG ATG GTC CTC ATG CTG GCG GCC CTC TCC CAG CAC TGC
        Met Lys Leu Leu Met Val Leu Met Leu Ala Ala Leu Ser Gln His Cys
          1                                   10

        117         126         135         144         153         162
TAC GCA GGC TCT GGC TGC CCC TTA TTG GAG AAT GTG ATT TCC AAG ACA ATC AAT
Tyr Ala Gly Ser Gly Cys Pro Leu Leu Glu Asn Val Ile Ser Lys Thr Ile Asn
              20                                  30

        171         180         189         198         207         216
CCA CAA GTG TCT AAG ACT GAA TAC AAA GAA CTT CTT CAA GAG TTC ATA GAC GAC
Pro Gln Val Ser Lys Thr Glu Tyr Lys Glu Leu Leu Gln Glu Phe Ile Asp Asp
                    40                                      50

        225         234         243         252         261         270
AAT GCC ACT ACA AAT GCC ATA GAT GAA TTG AAG GAA TGT TTT CTT AAC CAA ACG
Asn Ala Thr Thr Asn Ala Ile Asp Glu Leu Lys Glu Cys Phe Leu Asn Gln Thr
                          60                                          70

        279         288         297         306         315         324
GAT GAA ACT CTG AGC AAT GTT GAG GTG TTT ATG CAA TTA ATA TAT GAC AGC AGT
Asp Glu Thr Leu Ser Asn Val Glu Val Phe Met Gln Leu Ile Tyr Asp Ser Ser
                                    80

        333         342         351         360         369         378
CTT TGT GAT TTA TTT TAA CTT TCT GCA AGA CCT TTG GCT CAC AGA ACT GCA GGG
Leu Cys Asp Leu Phe ***
     90

        387         396         405         414         423         432
TAT GGT GAG AAA CCA ACT ACG GAT TGC TGC AAA CCA CAC CTT CTC TTT CTT ATG

        441         450         459         468         477         486
TCT TTT TAC TAC AAA CTA CAA GAC AAT TGT TGA AAC CTG CTA TAC ATG TTT ATT

        495
TTA ATA AAT TGA TGG CA 3'
```

Figure 2

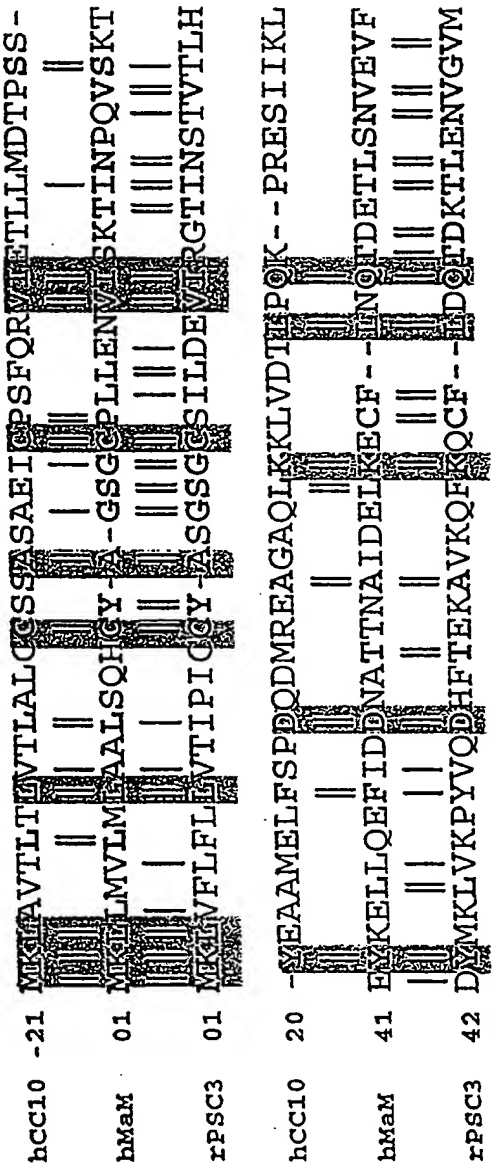

Figure 3

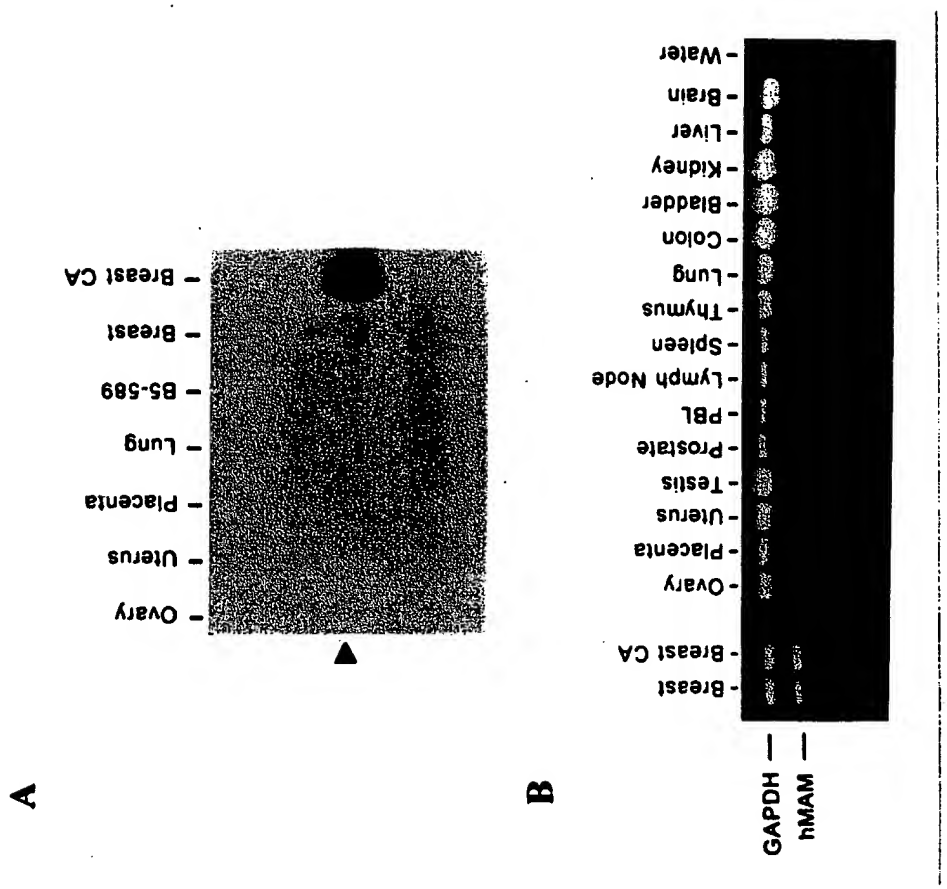
A
Ovary
Uterus
Placenta
Lung
BS-589
Breast
Breast CA
B
GAPDH
hMAM
Breast
Breast CA
Ovary
Placenta
Uterus
Testis
Prostate
PBL
Lymph Node
Spleen
Thymus
Lung
Colon
Bladder
Kidney
Liver
Brain
Water

Figure 4

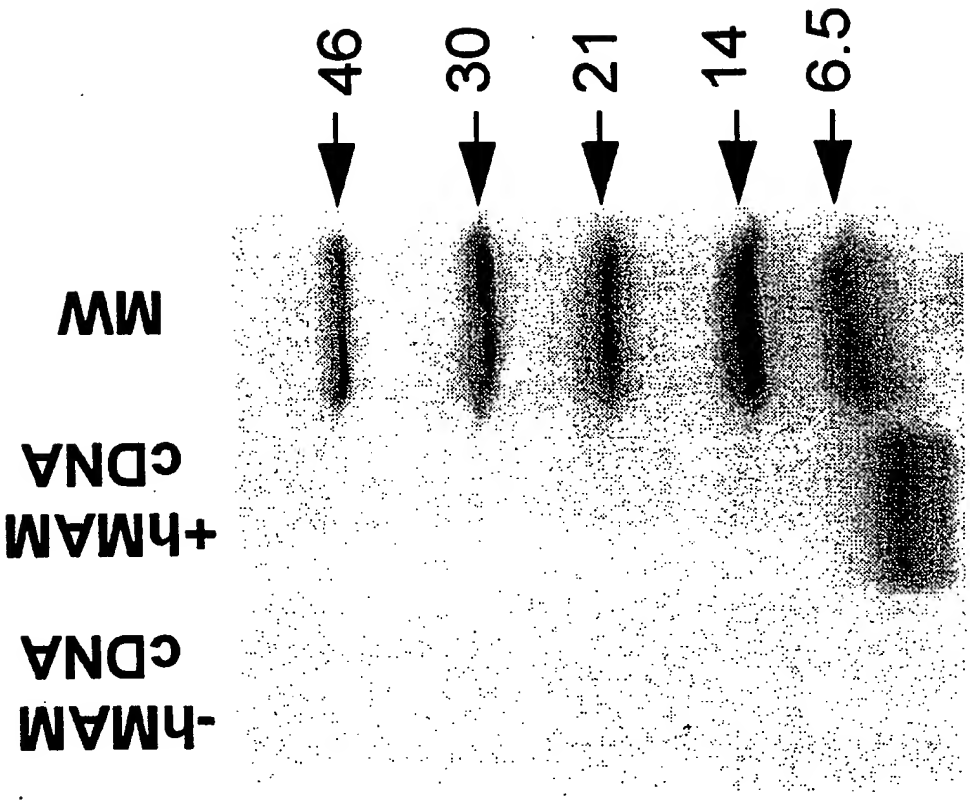

Figure 5

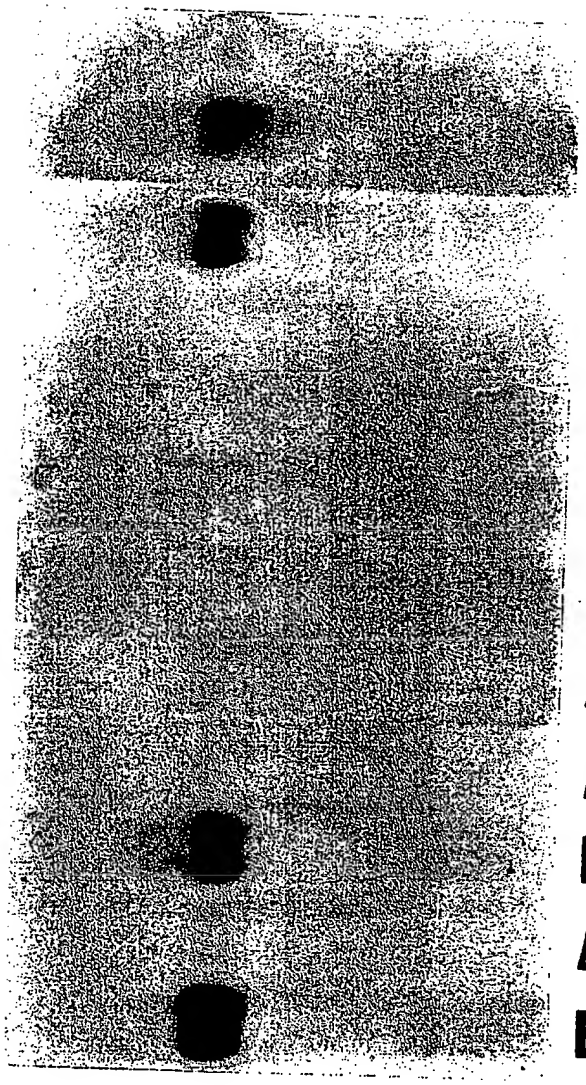

Figure 6

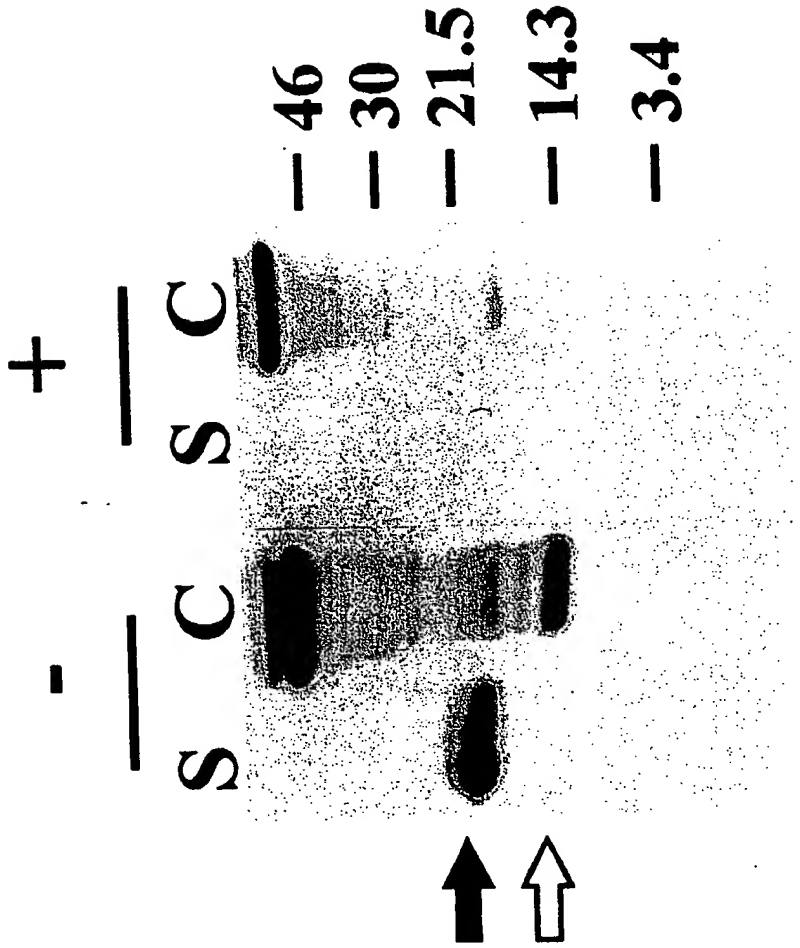

Figure 7

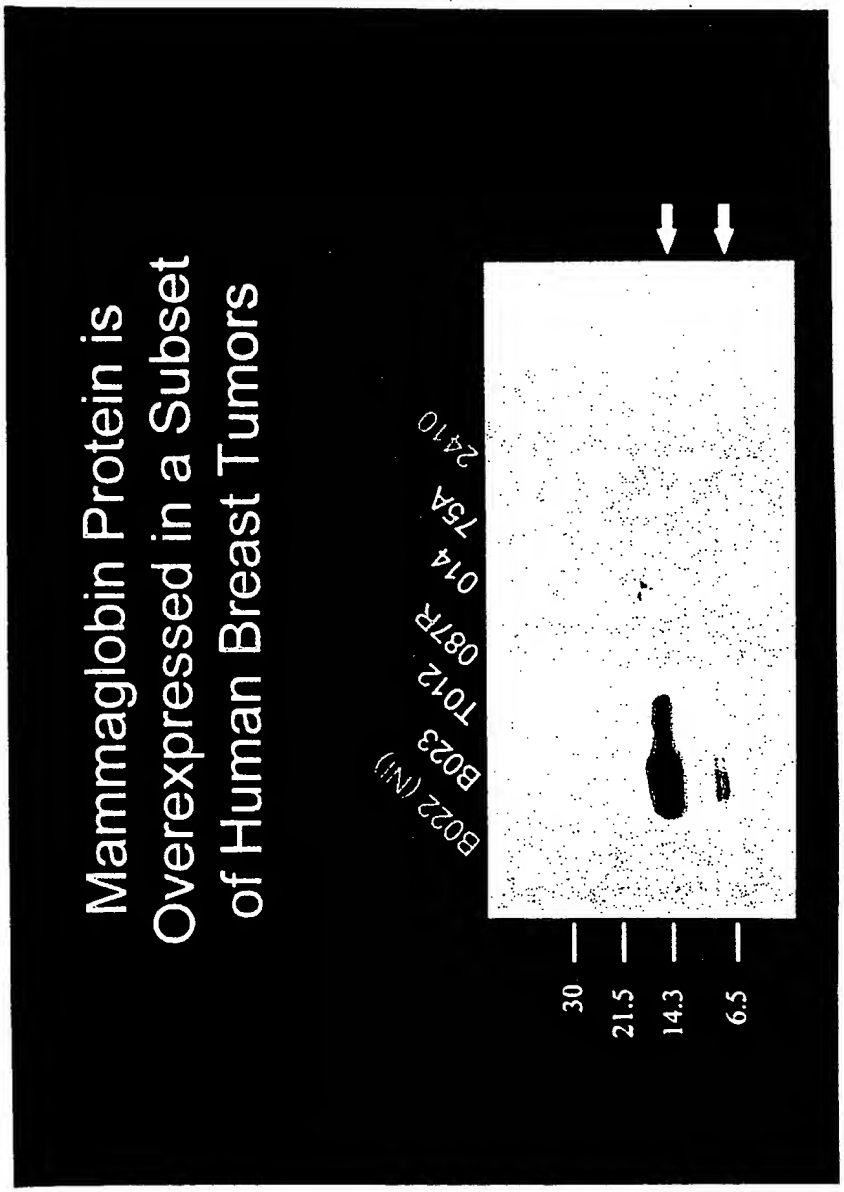
Mammaglobin Protein is Overexpressed in a Subset of Human Breast Tumors
B022 (NL)
B023
T012
08TR
014
75A
2410
30
21.5
14.3
6.5

Figure 8

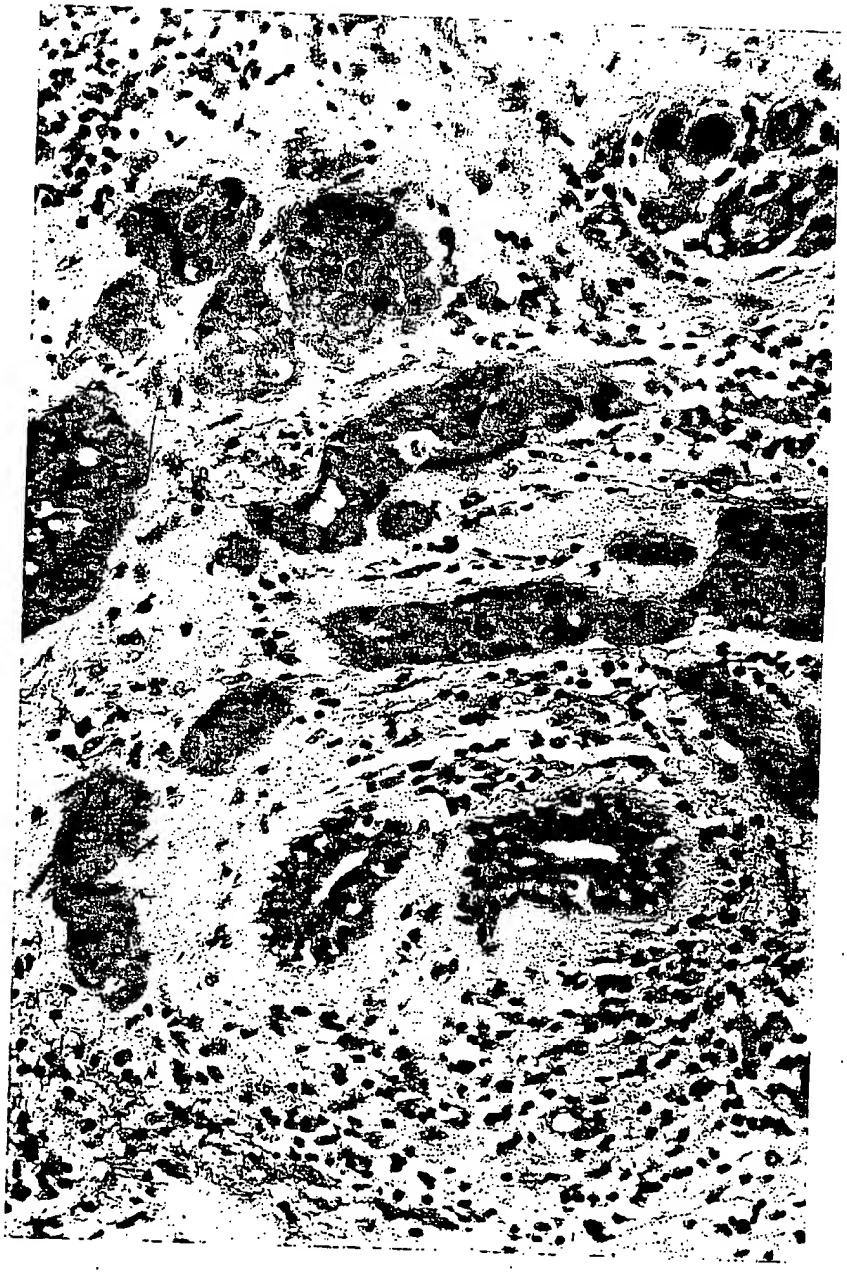

Figure 9

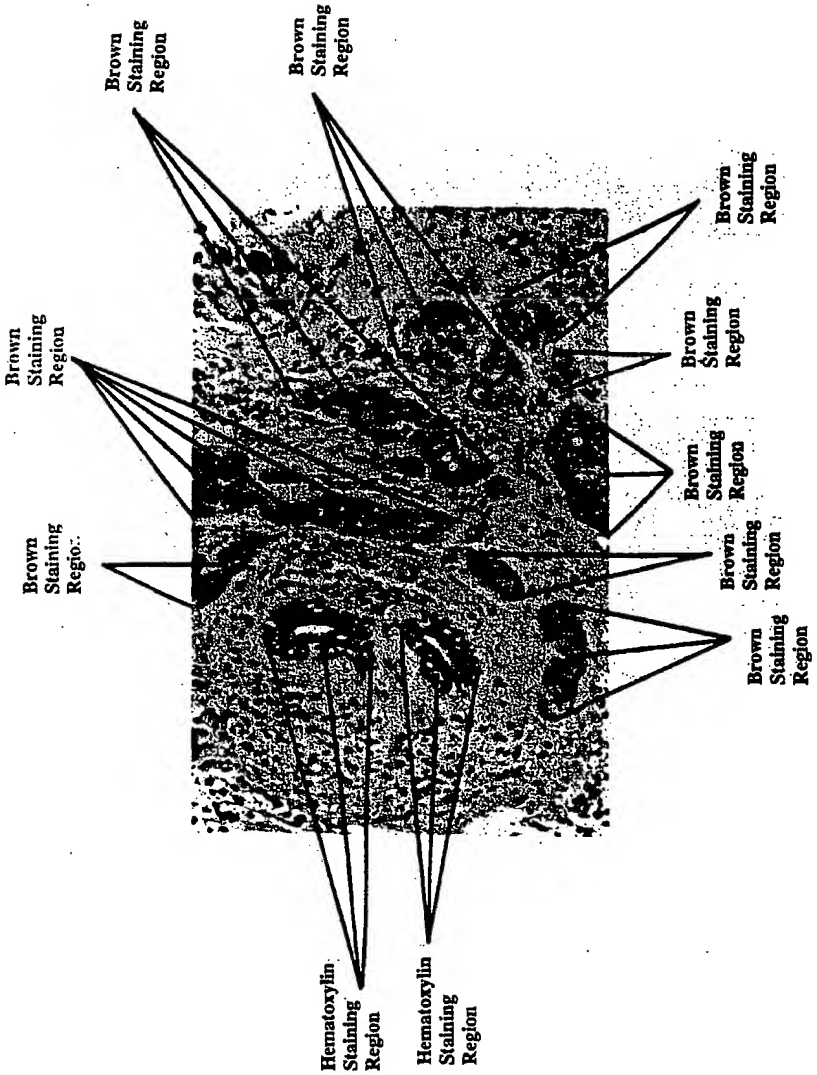
Brown Staining Region
Brown Staining Region
Brown Staining Region
Brown Staining Region
Brown Staining Region
Brown Staining Region
Brown Staining Region
Brown Staining Region
Brown Staining Region
Hematoxylin Staining Region
Hematoxylin Staining Region

Figure 9A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08235

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12Q 1/68; G01N 33/574
US CL : 536/23.5; 435/6, 7.23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/6, 7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIAGLOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Cancer Research, Volume 56, No. 4, issued 15 February 1996, M. A. Watson et al, "Mammaglobin, a Mammary-specific Member of the Uteroglobin Gene Family, Is Overexpressed in Human Breast Cancer", pages 860-865, especially page 861.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 JULY 1996	14 AUG 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
YVONNE EYLER

Telephone No. (703) 308-0196